Effects of supplementation with free radical scavengers on the survival and fertilization rates of mouse cryopreserved oocytes

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BACKGROUND: This study was conducted to investigate the effects of supplementation with free radical scavengers on the survival and fertilization rates of freeze–thawed mouse oocytes. METHODS: Superovulated oocytes with cumulus cells were cryopreserved by slow freezing in propanediol combined with a rapid thawing protocol. The cryopreservation medium was supplemented with the antioxidant enzymes superoxide dismutase (SOD) and catalase, and with the nitric oxide (NO) scavenger, haemoglobin (Hb). RESULTS: The addition of 50 IU/ml SOD showed significantly higher survival and fertilization capabilities compared with control (P < 0.01). Oocyte survival was greatly increased by concomitant addition of SOD with 10 IU/ml catalase (P < 0.01). On the other hand, the NO donor (sodium nitroprusside) inhibited survival and fertilization rates (P < 0.05). Significantly decreased survival and fertilization rates were also observed following the addition of high concentrations (10⁻³ to 10⁻⁶ nmol/l) of the NO synthase inhibitor N⁶-nitro-⁵-arginine methyl ester (l-NAME). In contrast, significantly better oocyte survival and fertilization rates were also observed following the addition of high concentrations (10⁻⁷ nmol/l) of the NO synthase inhibitor N⁶-nitro-⁵-arginine methyl ester (l-NAME). Moreover, oocyte survival and fertilization rates were significantly promoted by the concomitant addition of SOD with Hb (P < 0.01). CONCLUSIONS: These results suggest that supplementation of free radical scavengers, particularly combinations of SOD with NO scavengers in freezing and thawing media, improved the post-thaw survival and fertilization rates of cryopreserved mouse oocytes.

Key words: cryopreservation/catalase/mouse oocytes/nitric oxide/superoxide dismutase

Introduction

Reactive oxygen species (ROS), including superoxide anions (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH⁻), can cause damage to DNA and induce lipid peroxidation which adversely affects membrane structure, fluidity and function (Freeman and Crapo, 1982). Oocyte plasma membranes are susceptible to attack by oxidants because the phospholipid of the plasma membrane contains a significant proportion of esterified polyunsaturated fatty acids (PUFA) which are particularly sensitive to oxidative reactions. It has been reported that lipid peroxidation of membrane phospholipid is adversely involved in embryo development. The addition of antioxidant enzymes such as superoxide dismutase (SOD) or catalase into the culture medium could prevent membrane lipid peroxidation and, therefore, facilitate embryo development in the mouse (Halliwell, 1987; Aitken and Clarkson, 1988; Halliwell and Gutteridge, 1989; Nasr-Esfahani et al., 1990; Legge and Sellens, 1991).

In recent years, the role of nitric oxide (NO) has become of great interest in the field of reproductive biology. NO is one of the ROS that is thought to be involved in a variety of physiological cell signalling processes. It is synthesized from l-arginine by NO synthase (NOS) in various tissues and cell types and three distinct isoforms of NOS have been identified in different cells. Recent reports demonstrated that murine preimplantation embryos express both inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) and actually produce NO, suggesting the involvement of NO in embryogenesis (Vega et al., 1996; Gouge et al., 1998; Lim and Hansel, 1998). NO interacts with O₂⁻ and generates peroxynitrite anion (ONOO⁻), OH⁻ and nitrogen dioxide (NO₂) (Beckman et al., 1990), and it is these free radicals that are thought to exert greater toxicity towards cells than the ROS from which they originated.

The cryopreservation of oocytes has become an attractive tool for the treatment of infertility since it is associated with relatively few ethical and legal problems and could be developed alongside oocyte banking. However, the post-thaw survival and fertilization rates of oocytes remains low. Although studies using metaphase II oocytes have shown that the damage associated with cryopreservation is multifactorial, maintaining the membrane structure and permeability are important to prevent oocyte cryodamage (Mazur et al., 1963).

Some reports have indicated that cryopreservation can dam-
The addition of antioxidant enzymes which protect against lipid peroxidation and that freeze–thaw stress can be modified by incubating the embryo in the presence of inhibitors of membrane lipid peroxidation (Tarin and Trounson, 1993). These results suggest the possibility that the process of cryopreservation induces the production of ROS or alters the antioxidant enzyme potential of oocytes, leading to lipid peroxidation of their plasma membranes and resulting in reduced survival potential by a perturbation of membrane structure and permeability. In addition, it is possible that loss of survival and fertilization competence following cryopreservation of oocytes is also mediated by the mechanism of the cytotoxic action of peroxynitrite and NO₂. However, the definitive effect of free radicals on oocytes during cryopreservation has not been determined and the cross-reactivity between superoxide and NO has also not been investigated.

In the present study, we investigated the effect of supplementation with antioxidant enzymes and a NO scavenger on the survival and fertilization rates of freeze–thawed mouse metaphase II oocytes, using an in-vitro culture system. Data are also presented to evaluate the effects of NO inducer and NOS inhibitor on survival and fertilization potential of cryopreserved mouse oocytes. Moreover, particular attention was given to demonstrate the cross-reactivity of superoxide and NO during oocyte cryopreservation by the concomitant addition of SOD with a NO scavenger.

Materials and methods

Chemicals and enzymes

Haemoglobin (Hb) (human), Cu, Zn–superoxide dimutase (bovine liver), catalase (bovine liver) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). The NO inducer sodium nitroprusside (SNP) and L⁵-nitro-L-arginine methyl ester (L-NAME) were obtained from Wako Pure Chemical Industries Ltd (Tokyo, Japan). Propanediol was obtained from Nakarai Chemical (Tokyo, Japan).

Oocyte collection

Oocytes were collected from superovulated 6–8 week old ICR mice maintained on a constant light/dark cycle. Superovulation was induced by i.p. injections of 5 IU of pregnant mare serum gonadotrophin and 5 IU of human chorionic gonadotrophin (HCG) administered 48 h apart. The oviducts were excised 14–15 h after HCG injection and were immediately placed in phosphate-buffered saline (PBS). Freshly ovulated oocytes were released into PBS by puncturing the ampulla of each oviduct under stereomicroscope (Olympus SZH II LD, Japan). Pooled cumulus–oocyte mass from three or four mice were washed twice in PBS and randomly allocated to each test group and control.

Freezing and thawing protocols

Dulbecco’s phosphate-buffered saline supplemented with 10 mg/ml BSA was used for oocyte handling and dilution of test reagents. In test groups, different concentrations of chemicals and enzymes were added in both freezing and thawing media with the same concentration in each experiment.

Oocytes with cumulus cells were exposed stepwise for 5 min each to 0.5 mol/l, 1.0 mol/l, 1.5 mol/l propanediol and for 15 min to 0.1 mol/l sucrose with 1.5 mol/l propanediol in PBS supplemented with 10 mg/ml BSA at the room temperature. Oocytes with cumulus cells were then transferred to plastic straws, placed in a controlled programmed freezer (Cryoembryo HP; Daido-Hoxan, Tokyo, Japan) and cooled at 2°C/min from ambient to the seeding temperature –7°C. After manual seeding, the temperature was lowered to –30°C at 0.3°C/min and finally to –180°C at a rate of 50°C/min. Oocytes were kept in liquid nitrogen for 1–15 days. Oocytes with cumulus cells frozen by the slow-freezing method were thawed rapidly in a 37°C water bath.

The oocytes with cumulus cells were released directly into three steps of dilution of cryoprotectant each for 5 min. After being held in the 0.1 mol/l sucrose solution for 5 min, the oocytes with cumulus were washed with PBS and then cultured in human tubal fluid (HTF) supplemented with 4 mg/ml BSA. Survival of the oocytes after a freeze–thaw cycle was assessed by morphological criteria under an inverted microscope at ×200 magnification. The oocytes were considered as morphologically viable if, after thawing and following incubation for 1–2 h, they showed homogeneous cytoplasm with an intact plasma membrane and zona pellucida. At 1–2 h after thawing, cumulus cells surrounding the oocyte were spontaneously dispersed, therefore it was easy to observe the oocyte cytoplasm, plasma membrane and zona pellucida.

IVF and culture conditions

For IVF, spermatozoa were obtained from cauda epididymides of mature (4–6 months old) ICR male mouse. Spermatozoa were released into modified Krebs–Ringer bicarbonate medium supplemented with 4 mg/ml BSA, by puncture of the epididymis with a hypodermic needle and were allowed to disperse for 10 min at 37°C. After dispersion, the spermatozoa at a final concentration of 1×10⁶/ml were incubated for 2 h to induce capacitation before addition to oocytes. After 8 h of co-incubation with spermatozoa, the oocytes were examined for the presence of male and female pronuclei under a stereomicroscope, and later confirmed by an inverted microscope. The fertilized embryos were transferred to the HTF medium supplemented with 4 mg/ml BSA and were incubated for 24 h to confirm fertilization by observation of subsequent development.

Statistical analysis

Each experiment was repeated at least four times and six to 10 animals were used in each group. Treatment effects were evaluated by one-way analysis of variance using Statview II program (Abacus Concepts Inc.). Differences between groups were considered statistically significant at P < 0.05.

Results

Effect of catalase and SOD on post-thaw survival and fertilization rates

The addition of catalase ranging from 10–1000 IU/ml in freezing and thawing media showed no significant effect on rates of post-thaw survival and fertilization compared with control group (Table I). However, treatment with 50 IU/ml SOD significantly increased (P < 0.01) oocyte survival and fertilization rates compared with controls. Oocyte survival was significantly enhanced (P < 0.01 to P < 0.05) when 10 IU/ml catalase was concomitantly added to each group with 5, 50 or 500 IU/ml SOD. Combined addition of 50 IU/ml SOD with 10 IU/ml catalase greatly improved (P < 0.01) fertilization rates compared with control and 50 IU/ml SOD alone (Table II).
Effect of SNP, L-NAME and Hb on post-thaw survival and fertilization rates

Low concentration of SNP (10^{-8} mol/l) showed no significant effect on survival and fertilization rates but 10^{-6} and 10^{-7} mol/l concentrations of SNP significantly inhibited oocyte survival and fertilization compared with control (Figure 1). In contrast, treatment with low concentration of L-NAME (10^{-7} mol/l) improved (P < 0.05) both oocyte survival and fertilization. However, high concentrations of L-NAME ranging from 10^{-3} to 10^{-6} mol/l decreased oocyte survival and fertilization rates in a dose-dependent manner although a significant inhibitory effect (P < 0.05) was only observed at 10^{-3} mol/l.

Table I. Effect of catalase on survival and fertilization rate of freeze–thaw oocytes

<table>
<thead>
<tr>
<th>Control</th>
<th>Catalase dose (IU/ml)</th>
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<tbody>
<tr>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>No. of experiments</td>
<td>7</td>
</tr>
<tr>
<td>Total oocytes</td>
<td>165</td>
</tr>
<tr>
<td>Survival oocytes (%)</td>
<td>38.8 ± 6.4</td>
</tr>
<tr>
<td>Fertilized oocytes (%)</td>
<td>20.3 ± 1.5</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Values in the columns are not significantly different.

Table II. Effect of superoxide dismutase (SOD) and SOD + catalase on survival and fertilization rates of freeze–thaw oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (IU/ml)</th>
<th>No. of experiments</th>
<th>Total oocytes</th>
<th>Survival oocytes (%)</th>
<th>Fertilized oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>10</td>
<td>241</td>
<td>37.8 ± 7.1</td>
<td>14.3 ± 1.7</td>
</tr>
<tr>
<td>SOD</td>
<td>5</td>
<td>10</td>
<td>222</td>
<td>37.8 ± 4.5</td>
<td>11.9 ± 0.9</td>
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<tr>
<td>50</td>
<td>9</td>
<td>205</td>
<td>53.2 ± 5.4</td>
<td>28.4 ± 3.1</td>
<td>a</td>
</tr>
<tr>
<td>500</td>
<td>8</td>
<td>190</td>
<td>51.6 ± 3.0</td>
<td>18.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>SOD + catalase</td>
<td>5 + 10</td>
<td>6</td>
<td>185</td>
<td>50.8 ± 3.3</td>
<td>24.5 ± 3.1</td>
</tr>
<tr>
<td>50 + 10</td>
<td>6</td>
<td>157</td>
<td>58.6 ± 2.4</td>
<td>45.7 ± 4.8</td>
<td>a</td>
</tr>
<tr>
<td>500 + 10</td>
<td>6</td>
<td>187</td>
<td>51.3 ± 3.0</td>
<td>17.7 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SEM. 

Discussion

In this study, we have demonstrated that supplementation of 50 IU/ml of SOD to the freezing and thawing media improved survival and fertilization of mouse oocytes. In contrast, we did not find any beneficial effects of addition of only catalase on survival and fertilization potential at the concentrations used in this study. Concomitant addition of catalase with SOD showed significantly higher survival rates. In addition, a significantly higher fertilization rate was observed with a combination of 10 IU/ml catalase and 50 IU/ml SOD.
One of the well-recognized targets of oxidative injury is peroxidation of lipids in the plasma membrane. It has been proposed that autoxidation of PUFA, particularly lipids in plasma membranes, results in the formation of prostaglandin-like compounds by a mechanism involving free radical-catalysed peroxidation of arachidonic acid (Pryor et al., 1976). This pseudoprostaglandin is highly unstable and ultimately may cause perturbation of the plasma membrane structure.

Thus, the oocyte cryodamage might be induced by the mechanism that lipid peroxidation of oocyte plasma membrane induced by the generation of free radicals during freeze–thaw process causes the modification of membrane structure. The deformation of oocyte plasma membrane might lead to perturbation of membrane function, including permeability, intracellular pH regulatory systems and other transporter systems (Uechi et al., 1997; Lane et al., 2000). Generation of ROS during cryopreservation may also cause DNA strand breakage. Several studies have demonstrated the association of ROS-mediated oxidative damage of spermatozoa with sperm function by measuring a biomarker of oxidative DNA damage, such as uric acid (Munne and Estop, 1993) and 8-hydroxydeoxyguanosine (Shen et al., 1999). Moreover, ROS-induced damage of DNA has been associated with DNA fragmentation in oocytes and spermatozoa through apoptosis (Lopes et al., 1998). Our finding that the addition of SOD improved survival and fertilization potential after thawing indicates that exogenous addition of SOD may scavenge the free radicals generated during the cryopreservation process, and might protect against cryodamage of oocytes.

The addition of catalase to the culture medium might,
theoretically, be beneficial but we were unable to demonstrate any effect of catalase supplementation during cryopreservation. This finding is consistent in part with the previous reports in which catalase did not affect zygote development in mouse and rabbit (Legge and Sellens, 1991; Foote et al., 1993). Endogenous production of H2O2 in oocytes is not inhibited by adding catalase to the culture medium, since catalase dose not seem to penetrate the zona pellucida (Nasr-Esfahani et al., 1990). Therefore, exogenous addition of catalase would not affect the intra-oocyte production of H2O2 during the cryopreservation process. Alternatively, glutathione peroxidase within the oocytes can suppress the effects of H2O2 production by decomposition of H2O2 to H2O and alcohols (El Mouatassim et al., 1999); this may minimize the beneficial effect of exogenous addition of catalase.

The higher concentrations of SOD obviate the beneficial effect of SOD, especially on fertilization rate. SOD catalyses the dismutation of superoxide radicals and produces H2O2. This reaction could provide protection for oocytes against cryopreservation injury when H2O2 does not accumulate to harmful concentrations. However, when production of H2O2 exceeds critical levels due to the addition of higher concentrations of SOD, accumulated H2O2 affects oocyte quality. An increased level of SOD activity, that is not counteracted by a corresponding increase of catalase and glutathione peroxidase, may cause imbalances in the reduction–oxidation system, resulting in an extremely high rate of conversion of superoxide to H2O2 that cannot be counteracted (Sabatini et al., 1999).

This process is highly toxic since hydroxyl radicals, the most active ROS, could be produced through the Fenton or Haber–Weiss reactions in the presence of SOD, H2O2 and heavy metal ions. This might induce the lipoperoxidative damage to the membrane, cytoskeletal structure and DNA strand breakage of oocytes. The beneficial effect of the concomitant addition of SOD and catalase on cryopreserved oocytes observed in this study further supports the concept that the proper balance of the reduction–oxidation system could be important to maintain viability.

The interaction between NO and ROS has been well documented. NO reacts with superoxide, resulting in the formation of OONO–, OH–, NO2 and singlet oxygen. These are amongst the most active ROS, and cause oxidation of membrane lipids and intracellular damage. In our study, we observed that high concentrations of NO (10−6 and 10−7 mol/l SNP) but not low concentration of NO (10−8 mol/l SNP) significantly inhibited oocyte survival and fertilization rates after freezing and thawing. The process of freeze–thawing may generate increased amounts of NO, and exogenous addition of NO donor in freeze–thaw media produces further increased amounts of NO. Thus, generation of higher than physiological levels of NO interacts with superoxide leading to excessive formation of OONO– and OH– that may be as embryotoxic.

Our results also demonstrated that the addition of high concentrations of L-NAME (10−3 mol/l) significantly impaired oocyte cryopreservation. In contrast, significant beneficial effects were observed at low concentration of NOS inhibitor (10−7 mol/l L-NAME). A previous report in mice and rats has shown that embryos cultured in medium containing NOS inhibitors were developmentally delayed (Biswa et al., 1998; Gouge et al., 1998). Taken together, it is likely that the physiological level of NO is required for oocyte survival and subsequent development. Addition of low concentrations of NOS inhibitor could reduce the excessively generated NO during cryopreservation, but NO concentrations below the physiologically required amount, due to addition of high concentrations of NOS inhibitor, may impair oocyte survival. This concept may be further supported by our findings that the addition of appropriate concentrations of NO scavenger (1 and 0.1 µg/ml Hb) showed increased survival and fertilization rates. High concentrations of NO scavenger may reduce the physiologically required NO and very low concentrations of NO scavenger may limit diffusion of toxic amounts of NO accumulated during cryopreservation.

We found significantly higher survival and fertilization rates of cryopreserved oocytes when 0.1 µg/ml Hb was concomitantly added with 5 and 50 IU/ml SOD. The rate of peroxynitrite formation depends upon the product of O2– and NO concentration; it increases 100-fold for every 10-fold increase in O2– and NO concentration. Therefore, relatively small increases in the rates of O2– and NO production during cryopreservation may greatly enhance peroxynitrite formation to reach cytotoxic levels.

Beneficial effects of concomitant addition of NO scavenger and SOD suggest that cross-reaction between superoxide and NO may be involved in cryo-injury of oocytes, and prevention of this cross-reaction may be a highly effective method to protect oocyte plasma membrane from the stress of cryopreservation.

In conclusion, the present study has demonstrated that mouse oocytes can be cryopreserved successfully by supplementation with antioxidative enzymes and NO scavengers by stabilizing the critical changes occurring at the oocyte plasma membrane, as a result of ROS. We postulate that SOD plus catalase in the freezing and thawing media may protect against an imbalance in the reduction–oxidation system. Furthermore, we suggest that peroxynitrite, formed by the interaction of O2– and NO, may exert a cytotoxic effect on mouse metaphase II oocytes during cryopreservation, and the concomitant addition of SOD with Hb significantly improves oocyte survival and fertilization rates. Further studies of antioxidative enzymes and NO scavengers may lead to a better understanding of the biochemical processes that occur during oocyte cryopreservation.

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Free radicals and oocyte cryopreservation


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